Isolation of single cells using SH800 cell sorter for downstream gene expression analysis cytometry

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Background

In this study we demonstrate the workflow of isolating single cells using the SH800 cell sorter followed by gene expression analysis of the sorted single cells using the Roche LightCycler® Nano and LightCycler® 96. Furthermore, using data from sorted single cells, we demonstrate the ability to integrate phenotypic information obtained by flow cytometry with single cell gene expression analysis.

Material & Methods

For all experiments Sony SH800 cell sorter was used for sorting and Roche LightCycler® Nano or LightCycler® 96 for qPCR analysis.

Cell sorting: SH800 was setup for sorting with 100 um sorting chip at 20psi. Breakoff point was setup and maintained automatically by Core Finder™. Using the single cell sorting mode, defined number of cells were sorted into 1 ul of RNA lysis buffer in a cooled 96 well PCR plate. During sorting, index sorting feature was enabled.

Gene expression analysis: Cells were sorted into lysis buffer and incubated for 5 minutes at room temperature. RNA was extracted using RealTime ready Cell Lysis Kit (Roche). RNA was reversely transcribed to cDNA with Transcriptor Universal cDNA Master (Roche). For cDNA synthesis, cDNA synthesis mix was added into each well of sorted 96 well plate which contained lysate. The resulting cDNA was combined with PCR mix to perform qPCR with standard PCR protocol as per manufacturers guidelines. Measurement of GAPDH expression level was performed with RealTime ready Catalog Assays Human GAPDH (Roche) and FastStart Essential DNA Probes Master (Roche).

Results

Experiment 1: Accuracy of single cell sorting using SH800 Sorting strategy

-Freshly harvested Jurkat cells were resuspended in PBS and sorted using a SH800 cell sorter equipped with a blue laser. FSC-A was used as a trigger (0.5% threshold) and target population was identified by hierarchical gating on scatter plot (FSC-A vs. SSC-A) and singlets plot (FSC-A vs. FSC-H). Using the Sony SH800 defined number (1, 5, 10 or 50) of Jurkat cells were sorted into 1 ul buffer present in each well of a 96 well PCR plate .

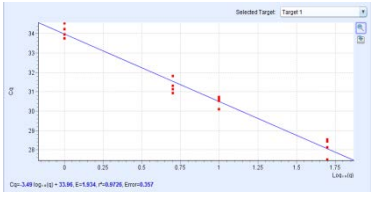
Cell lysis and qPCR analysis

-Post sorting, the RNA from individual Jurkat cells was extracted and qPCR analysis was performed to measure the expression of the GAPDH housekeeping gene on a per cell basis.

Results

-qPCR data showed strong correlation between number of sorted cells and Cq values (r^2 = 0.9726) and good efficiency (E=1.934, ideally 2.0) for each of the amplification plots of GAPDH gene.

(A)



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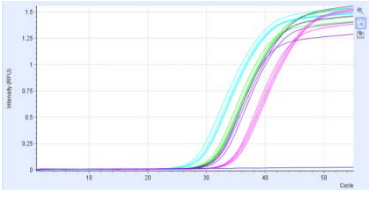


Figure 1: Accuracy of single cell sorting using SH800

Accuracy of single cell sorting was based on efficiency (E) and coefficient of correlation value (r^2) of qPCR results calculated on 1, 5, 10 and 50 sorted cells.
(A) Standard curve shows E=1.934 r^2=0.9726.
(B) Amplification curve of 50 cells (light blue), 10 cells (Green), 5 cells (purple) and 1 cell (pink).

Experiment 2: Comparison of indexed sort data and single cell gene expression analysis

Cell staining and sorting

-To demonstrate index sort analysis capability of the SH800 software, we used a 1:1 mixture of Jurkat and EL4 mouse cell line stained with anti-human CD45-FITC (Sony) and anti-mouse CD45-APC (Sony) respectively. The mixed population was stained with propidium iodide (PI) to differentiate between live and dead cells. FSC-A was used as a trigger (0.5% threshold) and a scatter gate was set on the mixed cell population (FSA-A vs. BSC-A) and live cells were identified (PI-A vs. BSC-A). APC+ and FITC+ single positive cells were identified (FITC-A vs. APC-A) and singlet live cells were identified (FSC-A vs. FSC-H). The target population was identified by a Boolean OR gate for the single FITC positive and APC positive cells.
Single cells from the target population were sorted into 1ul of RNA lysis buffer of a 96 well PCR plate to determine GAPDH expression. Index sort analysis was performed to determine the phenotype of the sorted single cells.

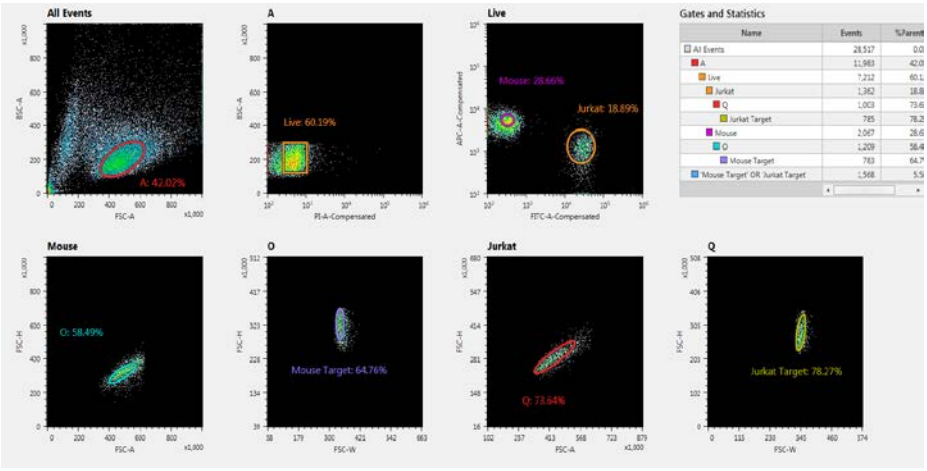
Cell lysis and qPCR analysis

-Post sorting, the RNA from individual cells was extracted and qPCR analysis was performed to measure the expression of the GAPDH (both human and mouse) housekeeping gene on a per cell basis. Measurement of human GAPDH expression level was performed with RealTime ready Catalog Assays Human GAPDH (Roche). Mouse GAPDH expression level was performed with Universal Probe Library Mouse GAPD Gene Assay (Roche) and FastStart Essential DNA Probes Master (Roche).

Results

- Using the index sort feature of the SH800 software, we could accurately determine the location of each sorted event from the Jurkat + EL4 mouse cell line mixture. The identity of the cells based on the flow cytometry data matched by 97% to that obtained by single cell gene analysis by qPCR.

(A)



(B)

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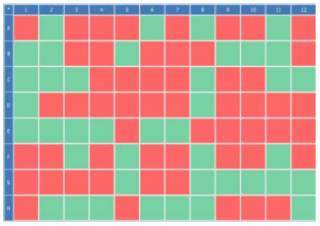

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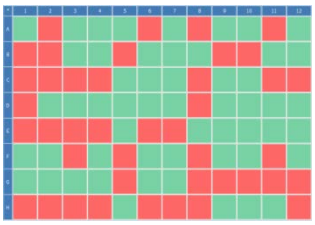


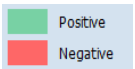


Figure 2: Comparison of indexed sort data and single cell gene expression analysis

(A)Sorting strategy for mixed population of Jurkat and EL4 mouse cells using the Boolean OR gate (Jurkat singlets OR Mouse singlets).
(B) Index data analysis, gate select mode, result of Jurkat cell.
(C) Index data analysis, gate select mode, result of EL4 mouse cells.
(D) qPCR result of Jurkat cell.
(E) qPCR result of EL4 mouse cell.

Experiment 3: Correlation between flow cytometry data and NFKB1 gene expression level in apoptotic cells

Cell staining and sorting

-Jurkat cells treated with DMSO only or PMA + Ionomycin (IO) for 1 hr. were stained with Annexin V (Sony) and propidium iodide (Sony) to analyze apoptosis stage of cells. FSC-A was used as a trigger (0.5% threshold) and a scatter gate was set on the live population (FSA-A vs. BSC-A) and live cells were identified as singlet cells (FSC-A vs. FSC-W).Cells in different stages of apoptosis, cells were identified apoptosis stage (FITC-A vs. PI-A), were sorted into 1ul of RNA lysis buffer of a 96 well PCR plate to determine NFKB1 gene expression by using the SH800.

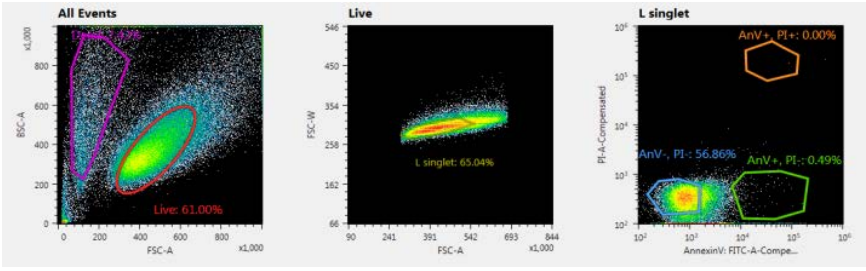
Cell lysis and qPCR analysis

-Post sorting, the RNA from individual cells was extracted and qPCR analysis was performed to measure the expression of the NFKB1 and GAPDH housekeeping gene on a per cell basis. To extract RNA, we used RealTime ready Cell Lysis Kit (Roche). RNA was reversely transcribed with Transcriptor Universal cDNA Master (Roche). Measurement of NFKB1 expression level was performed with RealTime ready Catalog Assays NFκB (Roche) measurement of human GAPDH expression level was performed with Universal Probe Library Reference Gene Assays, Human GAPD Assay (Roche) and FastStart Essential DNA Probes Master (Roche). qPCR was performed on the sorted single cells to determine the correlation between the level of NFKB1 expression and the apoptotic stage of the isolated cells. The cellular NFKB1 expression level was normalized to its GAPDH expression on a per cell basis.

Results

-By correlating the index sort analysis results of treated cells with NFKB1 expression, we could study the relationship between the apoptotic stage of cells and NFKB1 expression. According to the research of the past NFKB1 gene expression of Jurkat cells treated with PMA+IO after 1 hr. showed strong expression (ref 1). At first we sorted 10 cells per well and analyzed NFKB1 gene expression to reproduce previous data (ref 1). We observed strong expression by 1 hr. in PMA+IO stimulated cells. At 0 hr., cells treated with only DMSO showed lightly stronger expression in Annexin V+ PI- population than Annexin V- PI- population (Figure 3C). At single cell level, NFKB1 expression could not be detected significantly in cells at 0 hr. compared to higher levels seen post 1 hr. treatment. It is likely that the expression of NFKB1 in a single cell treated for 0 hr. is below the threshold of detection of qPCR system, but NFKB1 expression of 1 hr. exceeds the threshold (Figure 3D). As a positive control to check for sorting and qPCR efficiency, GAPDH gene expression analysis was performed for each well. We observed that most wells showed GAPDH gene expression with the exception of Annexin V+ PI- population (Figure 3E). Using index data analysis function of SH800 we studied the relation between phenotypic data and qPCR result. For this, we chose 3 cells for each treatment time point vs.
Annexin V- PI-,Annexin V+ PI- population (Figure 3F). One cell in 0 hr. and Annexin V- PI- population showed NFKB1 expression while no expression was detected in two other cells. The index data of these cells did not show significant difference (Figure 3G). We observe that the cells stimulated by PMA+IO showed stronger expression than the cells stimulated by only DMSO at single cell level.

(A)



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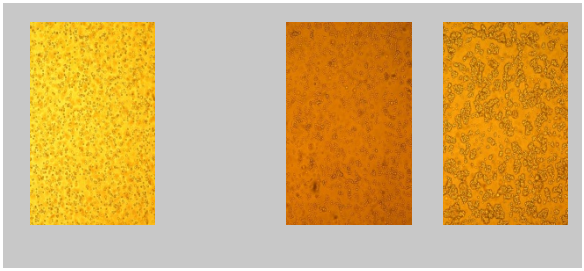


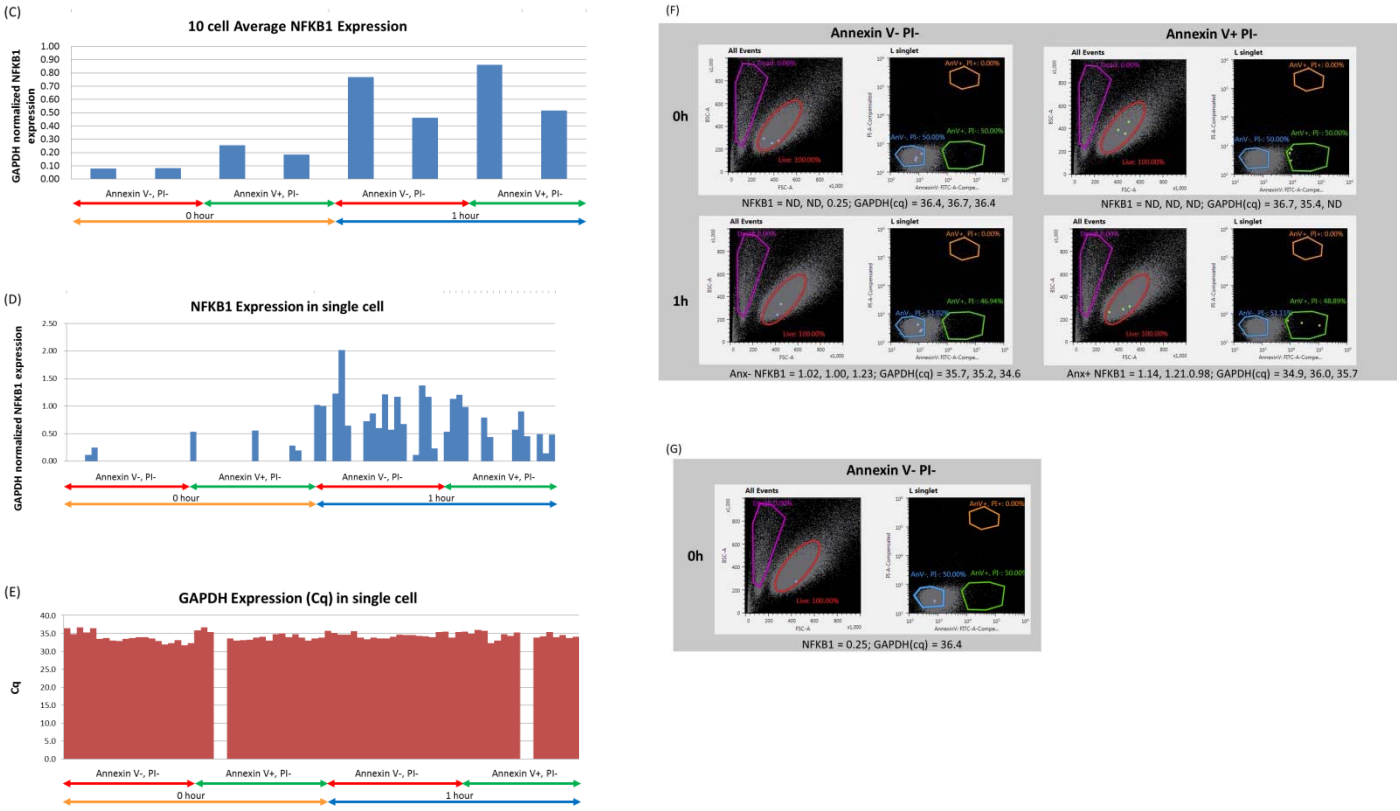
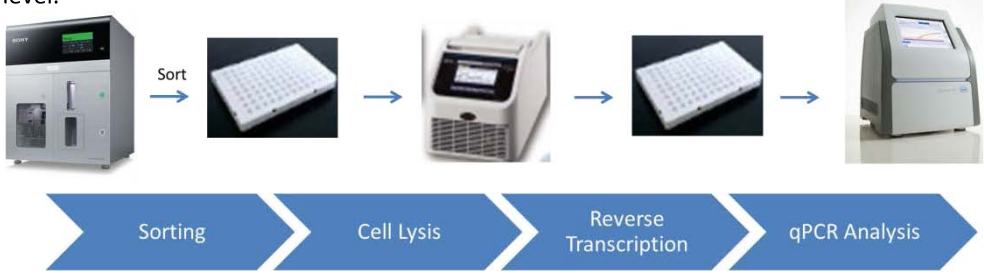


Figure 3: Correlation between flow cytometry data and NFKB1 gene expression level in apoptotic cells.

(A)Gating strategy for sorting Annexin V- PI- population and Annexin V+ PI- population.
(B) Image of cells to make sure stimulation of PMA+IO were working to cells.
(C) Average NFKB1 gene expression (n=10 cells). The data of Y axis was calculated by equation ((1/2^(NFKB1-Cq)))/((1/2^(GAPDH-Cq))).
(D) NFKB1 gene expression of single cells 0 hour and after 1 hour of stimulation with PMA+IO.
The data of Y axis was calculated by equation((1/2^(NFKB1-Cq)))/((1/2^(GAPDH-Cq))).
(E) GAPDH gene expression analysis of single sorted cells.
(F) Index analysis result for each conditions, 0 hour, 1 hour vs. Annexin V- PI-, Annexin V+ PI-.
(G) Index analysis result for 1 cell which NFKB1 gene expression detected in 0 hour and Annexin V- PI- condition.

Conclusion

In this study we showed a simple workflow for accurately sorting single cells using the SH800 cell sorter. Further, the index sort feature available in the software allows the end-user to study correlation between flow cytometry data and gene expression at a single cell level.



Reference

1. Chris Cheadle, Jinshui Fan, Yoon S Cho-Chung, Thomas Werner, Jill Ray, Lana Do, Myriam Gorospe and Kevin G Becker, Control of gene expression during T cell activation: alternate regulation of mRNA transcription and mRNA stability, BMC Genomics 2005,